OECD TEST GUIDELINES PROGRAMME

Standard Project Submission Form

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PROJECT TITLE

Guidance Document on Using Cytotoxicity Tests to Estimate Starting Doses for Acute Oral Systemic Toxicity Tests

SUBMITTED BY (Country / European Commission / Secretariat)

USA

DATE OF SUBMISSION TO THE SECRETARIAT

January 2009

DETAILS OF LEAD COUNTRY/CONSORTIUM

Country /Organisation:	USA
Agency/ministry/Other:	Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM)
Mail Address:	National Toxicology Program Interagency Center for the Evaluation of Alternative Methods (NICEATM) National Institute of Environmental Health Sciences NIH, DHHS P.O. Box 12233, MD: K2-16 Research Triangle Park, NC 27709
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PROJECT OUTCOMES

☐	New Test Guideline	☐	Guidance Document
☐	Revised Test Guideline	☐	Detailed Review Paper
☐	Deletion of an existing Test Guideline	☐	Other, please specify below

PROPOSED WORK PLAN and RESOURCE NEEDS:

1. Draft workplan for development of the proposal, including any need to establish Ad Hoc Expert Group and mode of meetings (face-to-face, teleconference; electronic discussion group). Indicate key milestones, including first and subsequent drafts of documents and timing of meetings.

The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) evaluated the use of two *in vitro* basal cytotoxicity test methods for estimating starting doses for acute oral systemic toxicity tests. This evaluation provides validation information that should be helpful to various stakeholders (e.g., applicable U.S. Federal regulatory agencies, the international regulatory community, the pesticide and other commercial chemical industries) in determining when these test methods might be useful for specific testing situations. Appropriate use of these *in vitro* test methods is expected to further reduce and refine animal use for acute oral systemic toxicity testing. The National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) and the European Centre for the Validation of Alternative Methods (ECVAM) organized an international, multi-laboratory validation study to evaluate the usefulness and limitations of two *in vitro* neutral red uptake (NRU) test methods. In this study, three laboratories tested 72 reference substances for basal cytotoxicity in BALB/c 3T3 mouse fibroblasts (3T3) and normal human epidermal keratinocytes (NHK). The resulting data were used to estimate starting doses for rodent acute oral systemic toxicity testing, based on linear regressions developed from the Registry of Cytotoxicity (RC; The German National Center for the Documentation and Evaluation of Alternative Methods to Animal Experiments [ZEBET]) database. The *in vivo* starting dose is determined from an LD_{50} value estimated by inserting the *in vitro* IC_{50} value into a regression formula that is derived from 282 substances for which there are both historical rat oral LD_{50} values and *in vitro* IC_{50} values from the RC. (http://iccvam.niehs.nih.gov/methods/acutetox/inv_nru_announce.htm)

ICCVAM considered recommendations from an international, independent expert review panel and from ICCVAM's advisory committee, the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM), as well as public comments received during the review process in making its recommendations on the use of the *in vitro* test methods. ICCVAM concluded that the 3T3 and NHK NRU test methods are not sufficiently accurate to predict acute oral systemic toxicity for the purpose of regulatory hazard classification. However, ICCVAM subsequently recommended that the 3T3 and NHK NRU test methods may be used in a weight-of-evidence approach to determine the starting dose for the current acute oral systemic toxicity protocols (i.e., the Up-and-Down Procedure [UDP], the Acute Toxic Class [ATC] method) which are described in OECD test guidelines 423 and 425. Data from the cell-based tests can then be used to identify the most appropriate starting dose to test in animals. For non-toxic substances estimated to be nontoxic, the in vitro methods can reduce the number of animals required for each in vivo test by as much as 50%.

A detailed summary of the comprehensive evaluation process that led to these recommendations is provided under **Item no. 7** below, and a summary of the information supporting these recommendations is included as **Attachment 1**. The information in this document will be based on the recently published ICCVAM recommended test method protocols (Stokes W, Casati S, Strickland J, Paris M. 2008. Neutral Red Uptake Cytotoxicity Tests for Estimating Starting Doses for Acute Oral Toxicity Tests. Current Protocols in Toxicology. May 2008. Suppl. 36: 20.4.1-20.4.20.) and included in the ICCVAM Test Method Evaluation Report (TMER, http://iccvam.niehs.nih.gov/methods/acutetox/inv_nru_tmer.htm).

To facilitate the greatest reduction in animal use, ICCVAM proposes to incorporate these recommendations on the use of the 3T3 and NHK NRU test methods in the proposed Guidance Document to encourage their use by OECD member countries. There is considerable interest

in use of in vitro methods in Europe due to the EU's impending 2009 ban on testing of cosmetic products on animals. In addition, the new European chemicals legislation, REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals), may require that thousands of existing chemicals be tested. This OECD Guidance Document will aid in reducing animal use relative to these programs. The proposed Guidance Document is scheduled for completion and submission to the Secretariat by March 2009.

2. Will additional information, including generation or collection of data, be required? If yes, please describe the anticipated process and timelines.

The proposed Guidance Document will be based on the NICEATM/ECVAM-sponsored international, multi-laboratory validation study of two *in vitro* neutral red uptake (NRU) test methods, as well as the technical evaluation and international independent peer review of the 3T3 NRU and NHK NRU test methods. Therefore, no additional data collection will be required.

3. Indicate the estimated overall resource need (time/money) for member country / consortium and Secretariat

The draft Guidance Document is scheduled for completion by March 2009, and resources for drafting the document will be provided by NICEATM. This effort will be coordinated with ICCVAM and the ICCVAM Acute Toxicity Working Group (OTWG), the European Centre for the Validation of Alternative Methods (ECVAM), and the Japanese Center for the Validation of Alternative Methods (JaCVAM). Following an initial round of commenting, if there is determined to be a need for an Expert Consultation to discuss any substantive issues, ICCVAM and NICEATM will coordinate and host the Expert Consultation.

4. Is this proposal intended to replace an existing Test Guideline or lead to the deletion of an existing Test Guideline?

No, the 3T3 NRU and NHK NRU test methods are intended to be used in conjunction with OECD GD 24 (Guidance Document on Acute Oral Toxicity Testing).

ESSENTIAL INFORMATION

In this section, please provide the information required by the Working Group of National Coordinators of the Test Guidelines Programme to assess the suitability of the project for the workplan of the Test Guidelines Programme

1. What is the existing or expected regulatory need/data requirement that will be met by the proposed outcome of the project? Please provide details below or as an attachment.
or as attachment No.__

The proposed NRU cytotoxicity test methods are to be used in a weight-of-evidence approach to determining the starting dose for acute oral systemic toxicity assays (i.e., the UDP and ATC method), which are used to provide such safety information. The default starting dose is typically used when there is no information upon which to base a starting dose (e.g., no toxicity information from chemicals with similar structure, etc.). U.S. and international regulatory agencies require safety testing to determine the potential hazards of products and chemicals. This information provides the basis for warnings on labels so that workers and consumers can take appropriate precautions to avoid injuries or illness.

2. How will the work contribute to further international harmonisation of hazard and risk assessment? Please provide details below or as an attachment.

Development of an OECD Guidance Document for these test methods will facilitate the consistent collection of data using standardized test method protocols. This will expand the current *in vitro/in vivo* validation database for the 3T3 NRU and NHK NRU test methods and periodic review will aid in further characterizing the usefulness and limitations of these methods. Such reviews will also aid in identifying where other in vitro test methods are needed to more accurately estimate the LD50 of test substances.

or as attachment No.__

3. How will the proposed project address issues and /or endpoints which are of major human health or environmental concerns? Please provide details below or as an attachment.

Acute oral toxicity testing is the most commonly conducted product safety test worldwide. It is vital for protecting public health by determining the poisoning potential of chemicals and products, so that the hazard potential of these substances is accurately identified and can be used to ensure proper labelling and packaging. However, the total number of animals used is large, and animals can experience significant pain and distress when test articles are toxic. Two internationally recognized test methods for acute oral systemic toxicity testing are the ATC and the UDP. Information on toxic doses and signs of acute toxicity can be obtained using either of these methods. Both methods are sequential tests in which the outcome of testing one or more animals at the first dose is used to determine the second dose that should be tested. In the overall strategy of hazard or safety assessment, the intended regulatory use of the *in vitro* NRU test methods is to reduce and refine the use of animals in current acute toxicity assays. However, they are not intended as complete replacements for the rodent acute oral systemic toxicity test methods.

or as attachment No.__

4. Will the project have general support from OECD member countries or is the outcome relevant for just one or a few member countries / stakeholders? Provide details of the countries and the rationale for this view below.

☐ Many countries ☐ A few countries ☐ Only for the submitting country

Given the national and international requirements for accurately labelling products with their poison potential, as well as global interest in further refining and reducing animal use for safety testing, it is anticipated that all member countries will support the proposed Guidance Document.

5. If the Test Guideline is not intended for general use, indicate if the Test Guideline would be intended for:

☐ Specific (limited) applications such as pesticide usage, or

☐ for specific classes of chemicals (e.g. surfactants) rather than for chemicals in general.

6. If the expected outcome of this proposal is a Test Guideline or a Guidance Document, provide information on the intended use, applicability and limitations of the test method.

The two in vitro cytotoxicity test methods are applicable to the OECD alternative acute oral systemic toxicity assays (the ATC and UDP), where the number of animals used depends on the starting dose. The number of dosing steps (and animals) is reduced if the starting dose is close to the true toxicity class (ATC) or the true LD_{50} (UDP). Animal savings are highest for chemicals with an estimated LD_{50} >5000 mg/kg. For less toxic chemicals, average animal use for the UDP was reduced by up to 22% per test and average animal use for the ATC method was reduced by up to 28% per test. The cytotoxicity methods are not likely to be appropriate for predicting starting doses for substances with toxic mechanisms that are not expected to be active in the two cell types, such as neurotoxicity and cardiotoxicity.

7. Provide supporting information on the validation status (i.e. relevance and reliability) of the method. Principles for validation of test methods for OECD Test Guidelines are described in Guidance Document 34.

Provide justification and rationale for the test, including data. If there are no or limited data available to support the reliability and relevance of the proposed test, indicate if validation work is included in the project. If there is no need for validation provide a detailed justification.

As indicated above, NICEATM and ECVAM completed a multi-laboratory validation study to evaluate the usefulness and limitations of the 3T3 NRU and NHK NRU *in vitro* test methods. **Attachment 1** provides a summary of ICCVAM test method recommendations on the *in vitro* test methods and the supporting information for these recommendations.

ICCVAM initiated a review of the validation status of *in vitro* methods for estimating acute oral toxicity in 1999 in response to a request from the U.S. Environmental Protection Agency (EPA) Office of Pesticides, Prevention, and Toxic Substances. The request was based on published studies that showed a correlation between *in vitro* and *in vivo* acute toxicity. An International Workshop on *In Vitro* Methods for Assessing Acute Systemic Toxicity was subsequently convened by ICCVAM and NICEATM in October 2000. Workshop participants concluded that the proposed *in vitro* methods had not yet undergone adequate studies to determine if they could meet regulatory requirements for acute toxicity testing.

NICEATM and ECVAM subsequently developed a collaboration 1) to further to characterize the usefulness and limitations of *in vitro* cytotoxicity assays as predictors of starting doses for rodent acute oral toxicity test methods, and 2) to develop a high quality database of *in vitro* cytotoxicity data that could be used to determine what other *in vitro* tests would be needed to accurately estimate acute toxicity hazard classification categories. NICEATM and ECVAM designed an international, multi-laboratory validation study to evaluate the performance of the 3T3 NRU and NHK NRU standardized *in vitro* test methods, using the ZEBET approach based on the Registry of Cytotoxicity (RC) regression model.

The validation study was initiated in August 2002 and completed in January 2005. Upon completion, NICEATM, in coordination with the ICCVAM Acute Toxicity Working Group

(ATWG) and ICCVAM, prepared a comprehensive draft background review document (BRD) containing the study results and analyses. ICCVAM subsequently convened an international independent Peer Review Panel (hereafter, Panel) meeting on May 23, 2006, to review the BRD, to evaluate the extent to which established validation and acceptance criteria had been addressed for the two methods, and to provide comments on draft ICCVAM recommendations on test method uses, future studies, draft test method protocols, and draft performance standards.

On July 11, 2006, an *FR* notice announced the public availability of and requested public comments on the *Peer Review Panel Report: The Use of In Vitro Basal Cytotoxicity Test Methods for Estimating Starting Doses for Acute Oral Systemic Toxicity Testing.* The Panel Report indicated that the information presented in the draft BRD was generally sufficient for its purpose. The Panel concluded that the applicable validation criteria were adequately addressed for use of these *in vitro* test methods in a weight-of-evidence approach to determine starting doses for acute oral toxicity tests.

The draft BRDs, Expert Panel report, and all public comments were subsequently made available to the ICCVAM's advisory committee, the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM). A teleconference working group meeting was held with the SACATM on August 3, 2006 to discuss the peer review panel's report and focus on the panel's conclusions regarding the draft ICCVAM recommendations for the proposed use of these test methods, draft test method protocols, draft performance standards, and draft recommended future studies. The SACATM concurred with the consensus conclusions of the Expert Panel. ICCVAM considered the peer review report, SACATM comments, and written public comments received on that report to prepare final ICCVAM recommendations for the two *in vitro* basal cytotoxicity test methods. An ICCVAM test method evaluation report (TMER), that included the final ICCVAM recommendations, was prepared and the ICCVAM and ATWG endorsed the report in October 2006. The final TMER was endorsed by the full SACATM in November 2006 and the TMER was forwarded to the appropriate federal agencies for their consideration. This report has been made available to the public and provided to U.S. Federal agencies for consideration, in accordance with the ICCVAM Authorization Act of 2000 (42 U.S.C. § 2851-2, 2851-5 [2000]) (Available at http://iccvam.niehs.nih.gov/methods/acutetox/inv_nru_tmer.htm). Agencies with applicable testing regulations and/or guidelines responded to ICCVAM within 180 days after receiving the ICCVAM recommendations. These responses were made available to the public on the ICCVAM website (http://iccvam.niehs.nih.gov).

ADDITIONAL INFORMATION

In this section please provide further information to allow the Working Group of National Coordinators of the Test Guidelines Programme to assess the suitability of the project for the workplan of the Test Guidelines Programme

1. If the expected outcome of the project proposal is a Test Guideline and is based on existing, regional or international documents such as guidelines, protocols or guidance material, please provide that information here or as an attachment.

Please see **Attachment 1** for supporting information used to establish ICCVAM recommended 3T3 NRU and NHK NRU test method protocols, upon which the Guidance Document will be based.

or as attachment No._1_

2. If Animal Welfare considerations are addressed in the project proposal, provide details below or as an attachment. Explain if the project is aimed at refining, reducing and/or replacing the use of animals.

If the project is not specifically developed for animal welfare purposes, indicate if the animal welfare considerations have been a component of the project proposal.

Indicate if animal welfare considerations are irrelevant to the project, for example for physico-chemical properties.

The use of *in vitro* basal cytotoxicity test methods were evaluated for estimating starting doses for acute oral toxicity tests to refine, reduce and/or replace the use of animals. The 3T3 NRU and NHK NRU test methods have been proposed as part of a weight-of-evidence approach to estimate the starting dose for acute oral *in vivo* toxicity test methods and should be considered and used where appropriate before testing is conducted using animals. For some types of substances, this approach will reduce the number of animals needed. In some testing situations, the approach may also reduce the numbers of animals that die or need to be humanely killed**.**

or as attachment No.__

3. Provide information on expected or possible resource savings in member countries as a result of this project.

Comparison of costs of GLP-compliant *in vitro* testing to *in vivo* testing is difficult because the *in vitro* NRU test methods are not replacements for the animal testing, which would be performed regardless of the responses of the 3T3 or NHK cells. The use of these *in vitro* NRU test methods may not reduce the overall cost of the *in vivo* rat acute oral toxicity test, but has the potential to reduce the number of animals needed for a study.

However, these test methods can provide a savings of time when used to determine if an *in vivo* acute oral toxicity limit test can be employed as the initial test for a substance with unknown *in vivo* toxicity. If the IC_{50} value from an *in vitro* NRU test accurately predicts an LD_{50} that is greater than, or equal to, the limit dose (i.e., 2000 mg/kg or 5000 mg/kg), then the *in vivo* test could start at the limit test dose. This approach has the potential to eliminate the need to do the main test and could result in a net savings of six days for the UDP test method and about one day for the ATC test method.

4. If the expected outcome of the proposed project is a Guidance Document or Detailed Review Paper, will it be directly linked to the development of a particular Test Guideline or a series of Test Guidelines?

☐ Yes, it is the initial step in the development of a new or revision of existing Guidelines.

☐ Yes, additional guidance is needed for the most appropriate selection of the Guidelines on the subject.

☐ No, the guidance is on issues related to testing or the development of Test Guidelines in general.

There is 1 attachment added to this form.

ATTACHMENT 1

BACKGROUND INFORMATION for the 3T3 NRU and NHK NRU *In Vitro* Basal Cytotoxicity Test Methods

In 2002, the National Toxicology Program Interagency Center for the Evaluation of Alternative Methods (NICEATM) and the European Centre for the Validation of Alternative Methods (ECVAM) initiated a collaborative, international, multi-laboratory validation study to independently evaluate the usefulness of the 3T3 and NHK NRU basal cytotoxicity test methods for estimating acute oral systemic rodent toxicity and for estimating starting doses for *in vivo* rodent acute oral systemic toxicity tests. The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) completed the technical evaluation of the test methods to determine the starting dose for the current acute oral toxicity protocols (i.e., the Up-and-Down Procedure [UDP], the Acute Toxic Class [ATC] method). The 3T3 and NHK NRU test methods were evaluated with 72 reference substances. Once the study was completed in January 2005, NICEATM prepared a draft background review document (BRD) that contained comprehensive summaries of the data generated in the validation study, analyses of the relevance and reliability of the two test methods, and simulation analyses of the refinement (i.e., to lessen or avoid pain and distress) and reduction in animal use that might occur if these tests were used as adjuncts to the UDP and ATC acute oral toxicity test methods. The draft BRD was released for public comment on March 21, 2006.

On May 23, 2006, NICEATM, on behalf of ICCVAM, convened an independent, scientific peer review panel meeting to review the draft BRD and evaluate the validation status of the 3T3 and NHK NRU test methods for determining starting doses for *in vivo* acute oral systemic toxicity tests. The peer review panel's report was released in July 2006. At a public teleconference meeting on August 3, 2006 the Scientific Advisory Committee on Alternative Toxicological Methods (SACATM) reviewed and endorsed the conclusions of the peer review panel.

ICCVAM considered the peer panel report, public comments, SACATM comments, and the draft BRD in finalizing its recommendations on the use of these two *in vitro* basal cytotoxicity test methods for estimating starting doses for acute oral systemic toxicity tests. The ICCVAM Test Method Evaluation Report (TMER) includes the ICCVAM recommendations on the use of the two *in vitro* NRU test methods, as well as recommended test method protocols, recommendations for future studies to further characterize the usefulness and limitations of *in vitro* methods for assessing acute systemic toxicity, recommended performance standards for tests with similar scientific principles and that measure or predict acute oral systemic toxicity, the peer panel report and *Federal Register* notices. The final BRD, which provides the supporting documentation for this report, is available as a separate document. The ICCVAM TMER and the supporting final BRD were forwarded to U.S. Federal agencies for their consideration for regulatory acceptance as required by the ICCVAM Authorization Act of 2000 (42 U.S.C. 285*l*–3).

The use of the NRU test methods to determine starting doses for acute oral systemic toxicity tests may reduce the number of animals required, and for relatively toxic substances, this approach may also reduce the number of animals that die or require humane euthanasia due to severe toxicity. This evaluation provides validation information that should be helpful to various stakeholders (e.g., applicable U.S. Federal regulatory agencies; the international regulatory community; the pharmaceutical, pesticide, and commercial chemical industries) in determining when these test methods might be useful and which test method might be the most appropriate for a specific testing situation. These *in vitro* test methods, when used appropriately, will reduce and refine animal use for acute oral systemic toxicity safety testing.

Recommendations Specific to the NRU Test Methods

There are sufficient data to support the use of the 3T3 NRU and NHK NRU test methods, in appropriate circumstances and with certain limitations, to estimate starting doses for *in vivo* acute

oral systemic toxicity testing. The 3T3 and NHK NRU test methods are not sufficiently accurate to predict acute oral toxicity for the purpose of regulatory hazard classification. For the purposes of acute oral toxicity testing, the 3T3 and NHK NRU test methods may be used in a weight-of-evidence approach to determine the starting dose for the current acute oral toxicity protocols (i.e., the UDP, the ATC method). For some types of substances, this approach will reduce the number of animals needed. In some testing situations, the approach may also reduce the numbers of animals that die or need to be humanely killed.

Limitations of the *in vitro* test methods include the inability of basal cytotoxicity to adequately account for mechanisms of action or evaluate absorption, distribution, metabolism, and excretion (ADME). The starting doses for substances with certain toxic mechanisms that are not expected to be active in 3T3 or NHK cells (e.g., those that are neurotoxic or cardiotoxic) will likely be underpredicted by these *in vitro* basal cytotoxicity test methods. Therefore, the results from basal cytotoxicity testing with such substances may not be appropriate for estimating starting doses.

The regression formula used to determine starting doses for test substances with known molecular weights and high purity should be the revised RC millimole regression line, based on substances with rat LD_{50} data, with IC_{50} values in mmol/L and LD_{50} values in mmol/kg. The regression formula used to determine starting doses for mixtures, test substances with low or unknown purity, or test substances with unknown molecular weights should be the revised RC regression line, based on substances with rat LD_{50} data, with IC_{50} values in mg/mL and LD_{50} values in mg/kg.

Compared to the NHK NRU test method, the 3T3 NRU test method appears to be less labor intensive and less expensive to conduct; therefore, the 3T3 NRU test method is recommended for general use. Although the 3T3 NRU test method was less reproducible than the NHK NRU test method, it produced slightly higher animal savings and accuracy for prediction of GHS acute oral toxicity category using the IC_{50} and the revised RC regressions evaluated for the prediction of LD_{50}.

When studies are conducted using this test method, the study protocol should be based on the recommended standardized 3T3 NRU and NHK NRU test method protocols provided in the ICCVAM TMER (see http://iccvam.niehs.nih.gov/methods/acutetox/inv_nru_tmer.htm). Exceptions and/or changes to the standardized test method protocol should be accompanied by a scientific rationale. Users should be aware that the 3T3 NRU and NHK NRU performance characteristics and the standardized test method protocols could be revised as additional data become available. For example, the current validation database did not allow for adequate evaluation of all chemical or product classes (e.g., mixtures, formulations). Additional data may allow for further evaluation of this, as well as other, chemical and product classes. Therefore, prior to initiation of 3T3 NRU and NHK NRU studies, investigators are encouraged to consult the ICCVAM/NICEATM website (see http://iccvam.niehs.nih.gov/methods/acutetox/acutetox.htm) to review the most current validation database, overall performance characteristics, chemical and physical class performance characteristics, and the recommended standardized test method protocols. Evaluation of the most current information will allow users to determine the appropriateness of this test method for evaluating substances that are within a specific chemical, physical, or product classes.

A detailed description of these recommendations can be found in the ICCVAM TMER: *In Vitro* Cytotoxicity Test Methods For Estimating Starting Doses For Acute Oral Systemic Toxicity Testing (available at http://iccvam.niehs.nih.gov/methods/acutetox/inv_nru_tmer.htm). The ICCVAM background review document for the *in vitro* basal cytotoxicity test methods contains a comprehensive summary of available data and information (Available at http://iccvam.niehs.nih.gov/methods/acutetox/inv_nru_brd.htm).

ASSESSMENT OF PROJECT PROPOSAL

(To be completed by all member countries /stakeholders except the submitter)

Country / Organisation:	

Representative: (Preferably NC):	

Taking into account the project information, requested above, does this project meet the needs of the member countries for addition to the workplan of the Test Guidelines Programme

☐ Yes ☐ No ☐ Further information needed

If the response is “No” or “Further information needed”, please provide justification:

Remarks as appropriate, including further information needs, if any:
